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Novel Approaches for Drug Targeting using Virosomes

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ABSTRACT

Virosomes are an innovative, validated and versatile adjuvant and carrier system with prospective applications for novel prophylactic and therapeutic vaccines against various diseases. There are reconstituted lipid vesicles equipped with viral glycoproteins are investigated as experimental vaccines for mucosal as well as systemic administration. Human parainfluenza viruses are extensively investigated and affinity-purified vesicles of hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins of human parainfluenza virus type 3 (P13 virus) were used to investigate their role in the induction of a protective immune response following immunization of hamsters. Results assured a complete resistance to challenged infection in case of intranasal administration on the other extreme partial protection was offered after subcutaneous immunization. Virosomes are advantageous as their surface glycoproteines possess high affinity for receptors on mucosal surfaces such as the respiratory tract thus providing a mechanism for effective liaison of antigens to mucosal surfaces. This review focuses on novel approaches for immunization using virosomes as a carrier system.

INTRODUCTION

Virosomes are spherical, unilamellar semi-synthetic complex derived from nucleic acid free viral particles with a mean diameter of 150 nm. They are essentially reconstituted viral coats, where a compound of choice replaces the infectious nucleocapsid. Virosomes retain their fusogenic activity and thus deliver the incorporated compound (antigens, drugs, genes) inside the target cell. They can be used for vaccines drug delivery or gene transfer [1-4].

Virosomes are small spherical vesicles containing viral membrane proteins (fusion proteins of the influenza virus) embedded in their lipid membranes. These proteins enable the virosomes membranes to fuse with cells of the immune system and thus deliver their contents - the specific antigens–directly to their target cells, eliciting a specific first-class immune response even with weak-immunogenic antigens. Once they have delivered the antigens, the virosomes are completely degraded within the cells [5, 6].

Virosomes are liposomes spiked with virus glycoproteins, incorporated into the liposomal bilayers based on retroviruses derived lipids (generally murine / avion). Reconstitution of a number of spike glycoproteins including those of sendai virus, rabies virus [7, 8], influenza virus, herpes virus, HIV-I and vesicular stomatis virus [9-12] into liposomes has been described. The virosomal constructs have been developed and used as a means of targeting to hamster hybridoma cells, murine lymph node T-cells, to elicit immune response in animals and in the mice and guinea pig [13, 14].

Commonly encountered problem with conventional liposomes is their internalization via endocytic compartment into the lysosomal system. To directly introduce molecules into the cytoplasm, liposomes that merge with cell membranes have been developed. The principle mimics the natural way by which several viruses bind and merge with cell membrane at neutral pH releasing their genome into the cytoplasm [15].

Development of virosomes exploits the ability of spike glycoprotein to undergo a conformational change at the endosomal pH, resulting in exposure of their hydrophobic residues to initiate fusion with plasma membrane of the cell or in case of small liposome, fusion with endocytic vacuoles. An alternative strategy to the reconstitution of virus spike glycoprotein in liposomes, involves the reconstitution of the virus receptor of the target cell contained in the liposomes. Fusion between the receptor bearing liposomes and the target cell is then brought about by the virus particle [16-20].

The virosomes may be target oriented and their fusogenic characteristics could be exploited in genome grafting and cellular microinjection. Proteoliposomes have been reported to deliver gene expression systems to cell. Liposomes prepared with fusogens (F) of sendai virus, incorporated into lipid bilayers (reconstituted fusogenic viral membrane) acquires character to merge with cell membrane [21-25].

Molecules with poor cellular membrane permeability [like the RNA duplex poly (rl). poly (rc)] have been very efficiently delivered to the cells with the help of fusogenic virosomes. Virosomes are recently reported as carriers for intracellular delivery of antisense

oligonucleotides have encapsulated oligonucleotides into negatively charged liposomes complexed to the protein core of an inactivated sendai virus. The approach exhibited a more rapid cellular uptake and higher transfection efficiency of oligonucleotides or plasmid DNA as compared to Lipofectin or passive uptake [26-30].

Virosomes have exhibited potential as immunological adjutants and as a result subsequent studies are directed towards their exploration in immunological manipulations [31-33].

ULTRASTRUCTURE OF VIROSOMES

Virosomes are spherical, unilamellar vesicles with a mean diameter of 150 nm. Essentially, virosomes represent reconstituted empty influenza virus envelopes, devoid of the nucleocapsid including the genetic material of the source virus. Virosomes are not able to replicate but are pure fusion-active vesicles. In contrast to liposomes, virosomes contain functional viral envelope glycoproteins: influenza virus hemagglutinin (HA) and neuraminidase (NA) intercalated in the phospholipid bilayer membrane. The unique properties of virosomes partially relate to the presence of biologically active influenza HA in their membrane. This viral protein not only confers structural stability and homogeneity to virosomal formulations, but it significantly contributes to the immunological properties of virosomes, which are clearly distinct from other liposomal and proteoliposomal carrier systems [34-38].

It has been shown that a physical association between the virosome and the antigen of interest is a prerequisite for the full adjuvant effect of virosomes. Such physical association can be achieved by a variety of methods, depending on the properties of the antigen. Antigens can be incorporated into virosomes, adsorbed to the virosome surface, or integrated into the lipid membrane, either via hydrophobic domains or lipid moieties cross-linked to the antigen [39-43].

ADVANTAGES OF VIROSOMES AS VACCINE PLATFORM

Virosomes fulfill all the prerequisites necessary to serve as new gold standard adjuvants and carrier systems for next generation prophylactic and therapeutic vaccines:

- The virus-like structure provides repetitive antigen presentation to B cells and mimics the natural presentation of antigen, which results in a specific and high-quality humoral immune response.
- The fully functional fusion-activity of virosomes enables receptor mediated uptake and natural intracellular processing of the antigen, which leads to the stimulation of both arms of the immune system: humoral and cellular immune responses.
- The antigen is partially protected from extracellular degradation and the resulting depot effect greatly facilitates Immunopotentiation.
- The flexibility of the virosome carrier allows the formulation and delivery of even barely soluble antigens.
- The two virosome-based vaccines already on the market show an excellent safety and side-effect profile.
- Virosome-based vaccines are suitable for infants and immunesuppressed persons.
- The virosomal technology platform is mainly composed of synthetic, well-defined biodegradable components and can be adapted to the specific requirements of any antigen of interest.
- Virosomes allow a patient-specific modular vaccine regimen [44, 45].

FUSOGENIC LIPOSOMES AND VIROSOMES

Fusogenic liposomes are specially engineered liposomes that fuse and merge with cell membranes and directly introduce molecules (entrapped or anchored) into cytoplasm thus avoiding the route followed by conventional liposomes, i.e., internalization via endocytic compartments into lysosomes.

The fusogenic liposomes mimic the way by which several viruses (HIV, sendai virus) bind and merge with cell membranes at neutral pH and subsequently release their genomes into the cytoplasm. The fusion between liposomes and cell membrane is not a spontaneous phenomenon and several methods are used to facilitate this type of delivery.

Virosomes are liposomes with virus spike glycoproteines incorporated into the liposomal bilayer. The spike glycoproteines of viral origin mediate the interaction of the virus with the target cell receptor. The interaction either results in the transfer of the virus genome into the host cell by a microinjection mechanism or results in membrane fission or fusion. Fusion can be mediated by fusogenic agents like polyethylene glycol, glycerol and polyvinyl alcohol (Table 1) or by reconstituted viral membrane based liposomes are also termed as Virosomes.

Fusion spike glycoproteins of sendai virus, rabies virus, measles virus, influenza virus, herpes virus, HIV-1 and vesicular stomatitis virus are incorporated in liposomes and these virosomes have been investigated for their immunoadjuvant, gene and oligonucleotide delivery system [46].

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Table 1. Methods used to facilitate fusogenic drug delivery. Viral spike glycoproteins and their functions.

Viral spike glycoproteins	Target site/ function
Sendai virus haemagglutinin/neuraminidase glycoprotein	Negatively charged liposomes
Rabies virus surface glycoprotein	Immune response in animals
Measles virus haemagglutinin and fusion glycoprotein	Murin macrophage cell lines
Influenza virus haemagglutinin/neuraminidase glycoprotein	Vaccine adjuvant
Herpes simplex virus glycoprotein D	Immune response in mice and guinea pigs
HIV-1 envelop glycoprotein	Immune response in mice lymphocytes

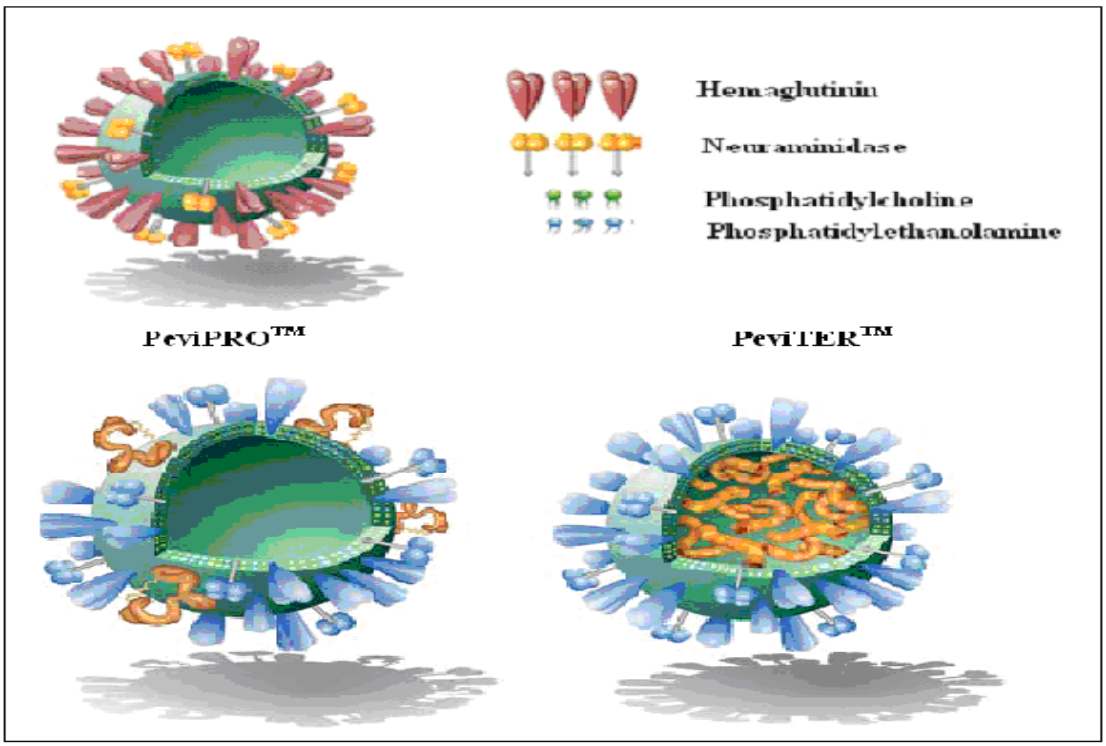

Figure 1. Virosomes: Influenza virus envelope with functional viral envelope of glycoprotein. Epitopes of the antigen are located on the surface of the virosome (PeviPRO™) or inside the virosome (PeviTER™) (from [47])

VIROSOMAL TECHNOLOGY PLATFORMS

Pevion uses its unique virosome-based technology platforms PeviPRO™ and PeviTER™ in the development of a new generation of vaccines characterized by eliciting a strong antigen specific immune response even in immunesuppressed humans. PeviPRO™ and PeviTER™ vaccines are composed of virosomes formulated with antigens of choice (peptides, proteins, carbohydrates or nucleic acids) (Figure 1). Depending on the localization of the antigens, the vaccines are able to evoke a predominantly humoral (PeviPRO™) and/or a cellular (PeviTER™) immune response, making them suitable as a prophylactic or a therapeutic vaccine [47].

VIROSOME IMMUNOPOTENTIATION MECHANISMS

The nature of the elicited immune response to virosome formulations is dependent on whether the epitopes of the antigen are located on the surface of the virosomes (PeviPRO™) or inside the virosome (PeviTER™). PeviPRO™ elicits a humoral immune response. The antigen is degraded in endosomes of the cell and therefore generates predominantly a MHC II antigen-presentation (Figure 2). PeviTER™ formulated antigens generate in vivo not only a CD4+ and CD8+ positive response but are also able to induce a strong cytotoxic T-cell response (CTL). Virosomal encapsulation ensures a proper presentation of the antigens through the MHC I pathway because the antigen is delivered in a natural way into the cytosol of the antigen presenting cell.

The influenza virus surface glycoprotein HA guides the virosomes specifically to antigen-presenting cells and leads to fusion with their endosomal membrane. This process provides optimal processing and presentation of the antigens to immunocompetent cells. The T lymphocytes are activated to produce cytokines, which in turn stimulate the B lymphocytes to form large amounts of specific antibodies. The stimulation of B lymphocytes also occurs through direct contact with the antigen-virosome complex [48, 49].

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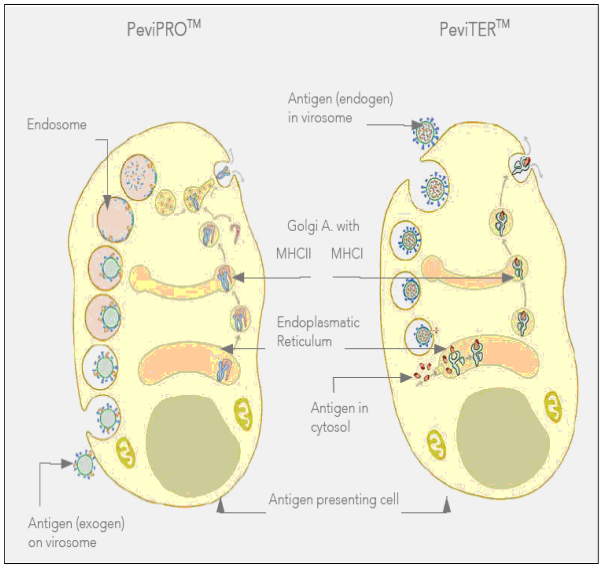

Figure 2. Virosome-based antigen processing**.** The localization of the antigen is the crucial factor (from [48]).

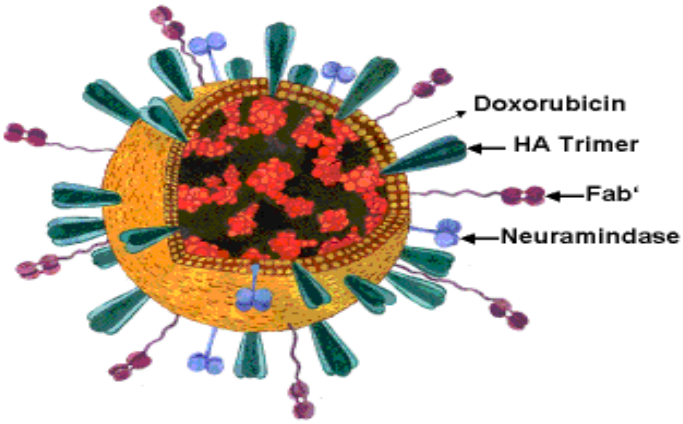

Figure 3. Doxorubicin targeting using virosome. Virosomes conjugated with an antibody (Fab' fragments of an anti-rNeu monoclonal antibody, mAb) against a tumor antigen (from [53]).

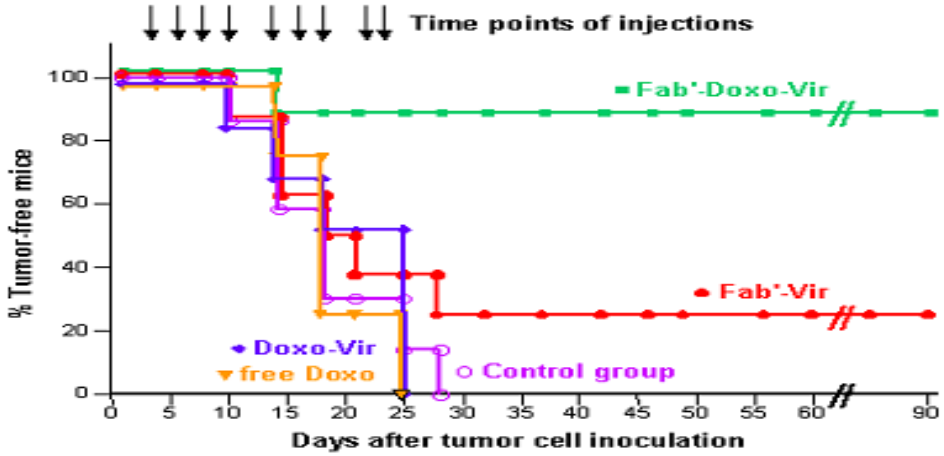

Figure 4. Effect of virosome treatment on recently implanted tumors. 2 x 10^5 $rNeu^+$ tumor cells were injected sub cutaneous into mice and treatment was started 3-5 days later. Intra venous injections of the different virosome formulations such as Doxo-Vir (blue line), Fab'-Doxo-Vir (green), Fab'-Vir (red) and free Doxo (orange) (all at a concentration of 150 µg/ml doxorubicin) were performed at the indicated time points (arrows) for a period of three weeks. Tumor size was assessed twice a week during therapy and weekly in the follow-up period. Tumor formation (defined as volume › 90 $mm^{3)}$ in the different groups and the follow-up of >12 weeks is shown (from [53]).

When tested for safety and immunogenicity in elderly nursing home residents in comparison to whole-virion and subunit vaccines, the virosome-formulated influenza vaccine was found to be superior with regard to reactogenicity and immunogenicity [50, 51].

THERAPEUTIC APPLICATION OF VIROSOMES

Virosomes in Oncology

Waelti *et al* used virosomes (reconstituted fusion-active viral envelopes) as a new drug delivery system and have shown that virosomes are capable of binding and penetrating into tumor cells, delivering cytotoxic drugs (Figure 3). Waelti *et al* have additionally demonstrated that conjugating Fab' fragments of an anti-rNeu monoclonal antibody (mAb) to virosomes selectively and efficiently inhibits tumor progression of established rNeu overexpressing breast tumors (Figure 4).

Fab'-Doxo-Virosomescombinethe antiproliferative properties of the mAb and the cytotoxic effect of doxorubicin in vivo (Figure 5). Furthermore, Fab'-Doxo-Virosomes significantly inhibit tumor formation at a tumor load representing metastatic spread (Figure 6) These results indicate that virosomes conjugated with an antibody against a tumor antigen are a promising new selective drug delivery system for the treatment of tumors expressing a specific tumor antigen [52, 53].

Virosomal vaccines

The induction of effective cellular and humoral immune responses against antigens is the major goal in vaccination strategies against infectious diseases and cancer. Influenza virosomes as a versatile delivery system for molecules of a different nature, such as proteins, peptides and nucleic acids. These molecules can be administered in different ways, such as nasal, muscular or dermal routes, also facilitating the uptake of the antigen by antigen-presenting cells (APC) and inducing a specific humoral and cell-mediated immune response, particularly a CTL response (Figure 7). Influenza virosomes, due to their applicability in different medical fields, are a promising tool in vaccinology and immunotherapy applications [54-58]. The particle structure, together with the functions of hemagglutinin-receptor binding, pH-dependent fusion activity and immunostimulation is responsible for the adjuvant effect of virosomes [59-61].

Virosomal subunit vaccine against malaria

It is generally assumed that an effective malaria vaccine has to target antigens of the different developmental stages of the parasite. They developed new methods to identify and optimize Peptidomimetics of malaria surface antigens (mimotopes) and to embed them into the surface of immunopotentiating reconstituted influenza virosomes (IRIVs). IRIVs are spherical, unilamellar vesicles prepared from influenza virus envelope glycoproteins and a mixture of natural and synthetic phospholipids. Influenza haemagglutinin (HA) intercalated into the liposomal bilayer plays a key role in the mode of action of IRIVs. HA mediates the binding of IRIVs to cell surface receptors and their subsequent fusion with the endosomal membrane, thus initiating a successful immune response through efficient MHC class I and MHC class II restricted antigen presentation. Antigens linked to the surface of virosomes are released within the phagolysosome upon endosomal fusion, and the associated epitopes are thereby presented to CD4 T cells by MHC class II molecules [62-65].

Antigens encapsulated in the virosomes are delivered to the cytosol during the fusion event, and will enter the major histocompatibility complex (MHC) class I pathway. IRIVs are there-fore able to induce both a CD4 T/B cell and a CD8 T cell immune response. The first two registered IRIV-based vaccines in use are a virosomal hepatitis A vaccine and a trivalent influenza vaccine; both show excellent immunogenicity and safety profiles. Antigen delivery using IRIVs thus represents an attractive alternative to aluminium salt-based adjuvants [66]. One of the main difficulties in designing such mimotopes lies in the unknown conformation of the surface loops of most of the potential target proteins. With the ViroTope approach we can optimize mimotopes in an iterative development process (Figure 8). Using this approach, we have identified and optimized synthetic peptide structures that mimic the surface loops of two candidate malaria vaccine antigens:

- NPNA repeat region of the circumsporozoite protein (CSP),
- Loop I of domain III of merozoite apical membrane antigen-1 (AMA-1).

Lead structures of additional antigens have been identified and additional optimized mimotopes are being developed.

Virosomal HIV vaccines

A safe and effective vaccine is the only way to stop the spread of HIV, especially in developing countries. Ferko Boris suggested an alternative approach in HIV vaccine development: to test virus-like particles composed of primary HIV proteins or recombinant proteins with native conformation, which are incorporated into liposomes of the size of virions and with a lipid composition similar to the virus membrane. Resulting virosomes carrying HIV proteins will be utilized as prophylactic and therapeutic vaccine against HIV-1 infection.

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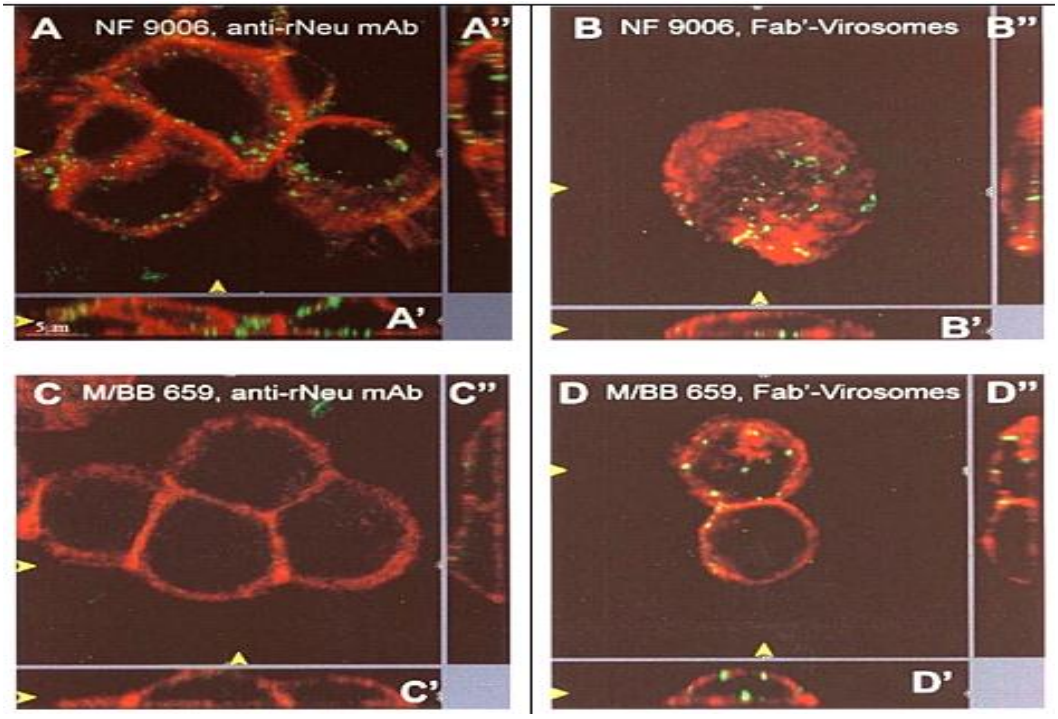

Figure 5. Analysis of internalization in confocal laser scanning microscopy (CLSM). rNeu$^+$ and rNeu$^-$ tumor cells were stained with FITC-labeled anti-rNeu mAb (A+C) or FITC labeled Fab'-Vir (B+D, green) and rhodamine-labeled F-actin (red). NF 9006 cells are rNeu$^+$, M/BB 659 cells are rNeu$^-$. 3D reconstruction: xy-projection (A', B', C', D'), xz-projections (A'', B'', C'', D''). Arrows mark the position of projections (from [53]).

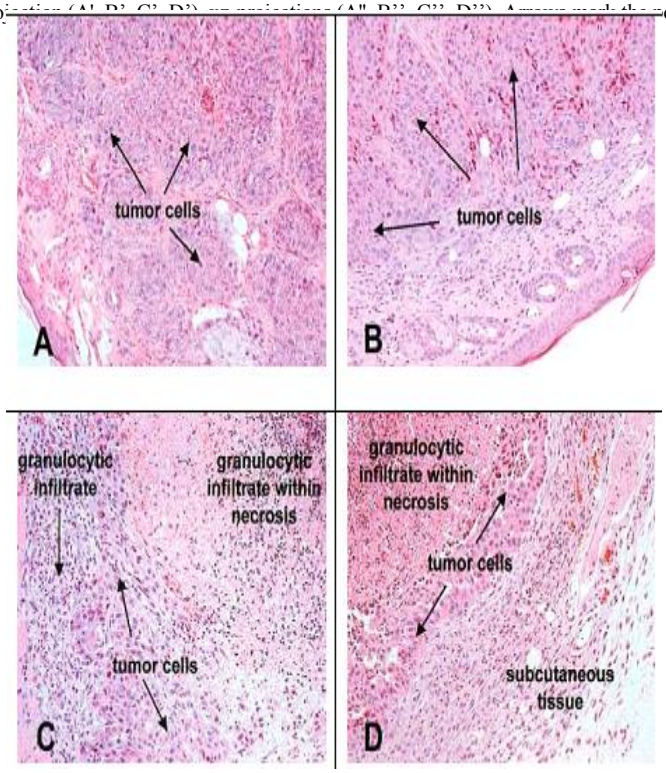

Figure 6. Histological analysis of rNeu$^+$ tumor injection site. Paraffin sections from tumor injection site at day 5 after injection of $2x10^6$ rNeu$^+$ tumor cells (line NF9006) into
A) control animals, B) mice treated with Doxo-Vir, C) mice treated with Fab'-Vir and D) mice treated with Fab'-Doxo-Vir. Treatment was performed every 2nd day (from [53]).

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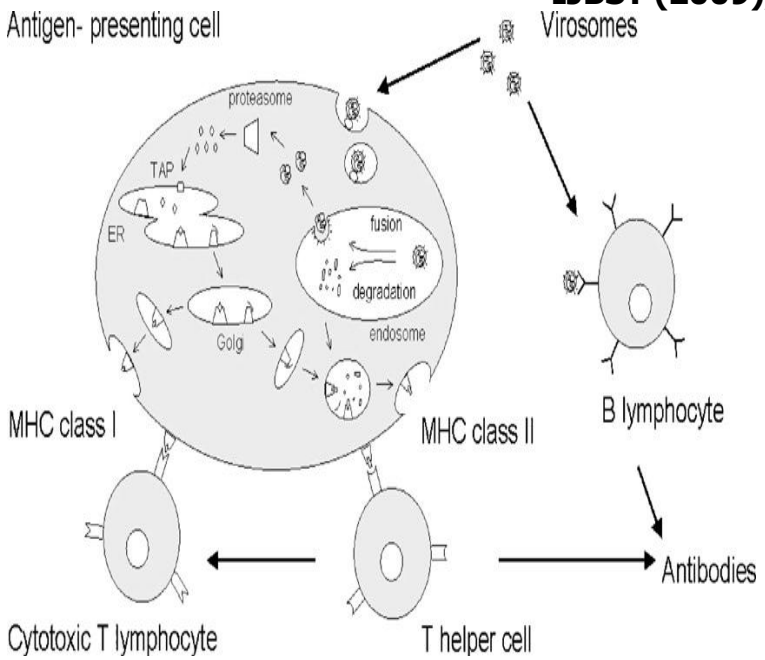

Figure 7. Putative interactions of virosomes with cells of the immune system. By virtue of the repetitive arrangement of haemagglutinin on the virosome surface, virosomes interact efficiently with immunoglobulin receptors on B lymphocytes. Virosomes are also taken up avidly by antigen-presenting cells, particularly dendritic cells. Antigens on the virosome surface, as well as antigens derived from degraded virosomes, enter the MHC class II pathway, activating T helper cells. Antigens inside the virosomes, through fusion of the virosomes, access the cytosolic MHC class I presentation pathway, activating cytotoxic T lymphocytes (CTL) (From [55 and 56]).

Since this vaccine is cheap to produce, is stable and might be administered in a needle-free format, this approach ideally meets the specific needs of developing countries. A new US patent invention provides Alphavirus vectors and virosomes with modified HIV genes for use in vaccines [67, 68].

VIROSOMAL GENE DELIVERY AND ANTISENSE STRATEGIES

Virosomes with a positively charged lipid bilayer (cationic) have been developed for transfer of genetic material. The positively charged lipid bilayer interacts with nucleic acids and forces it to concentrate within the vesicles formed. The vesicular stomatitis virus (VSV) glycoprotein (G) was used to prepare virosomes as a model vehicle of gene transfer to animal cells, for which viral envelope functions (receptor recognition and binding and the pH-dependent membrane-fusion) were expected to work.

Antisense oligodeoxynucleotides (ODN) are short nucleotide sequences of DNA synthesized as reverse complements of the nucleotide sequence of the target mRNA. On formation of the RNA-DNA duplex, translation of the message is prevented and the destruction of the molecule by RNase H is promoted.

Delivery of ODN targeting oncogene-encoded mRNA to cancer cells may be associated with inhibition of cell proliferation and, in some circumstances, cell death. Antisense ODN have a great potential as therapeutic agents. Several new strategies have been presented in the recent past for correction of genetic diseases of muscle and skin. Encouraging reports have been published in delivering genes encoding liver-derived factor IX to correct hemophilia B and fumarylacetoacetate hydrolase to cure hereditary tyrosinemia type I. Serious attempts to target genes to precise locations (such as airway epithelial cells of the respiratory tract) in diseases such as cystic fibrosis have so far proved to be difficult and exemplify the need for new delivery methods. Newer viral and nonviral vectors/vehicles, which hold promise to facilitate target-specific vector uptake and retention and are able to evade unwanted immune responses, constitute the major demanding criteria for durable and successful gene therapy for genetic diseases [69-71].

Addition of cationic virosomes (75-150 µl) containing antisense L-myc OPT in the picomolar range to small-cell-lung-cancer (SCLC) cell cultures that expressed highly the L-myc oncogene led to strong inhibition of thymidine incorporation in a concentration-dependent manner. Virosome-entrapped sense L-myc OPT and random-order OPT had only minimal effects on the thymidine uptake. In Western-blot analysis, expression of L-myc protein was suppressed in the antisense-virosome-treated NCI-H209 cells but not in untreated control NCI-H209 cells. These results suggest that cationic virosomes may have great potential as an efficient delivery system for antisense oligonucleotides in cancer therapy [72, 73].

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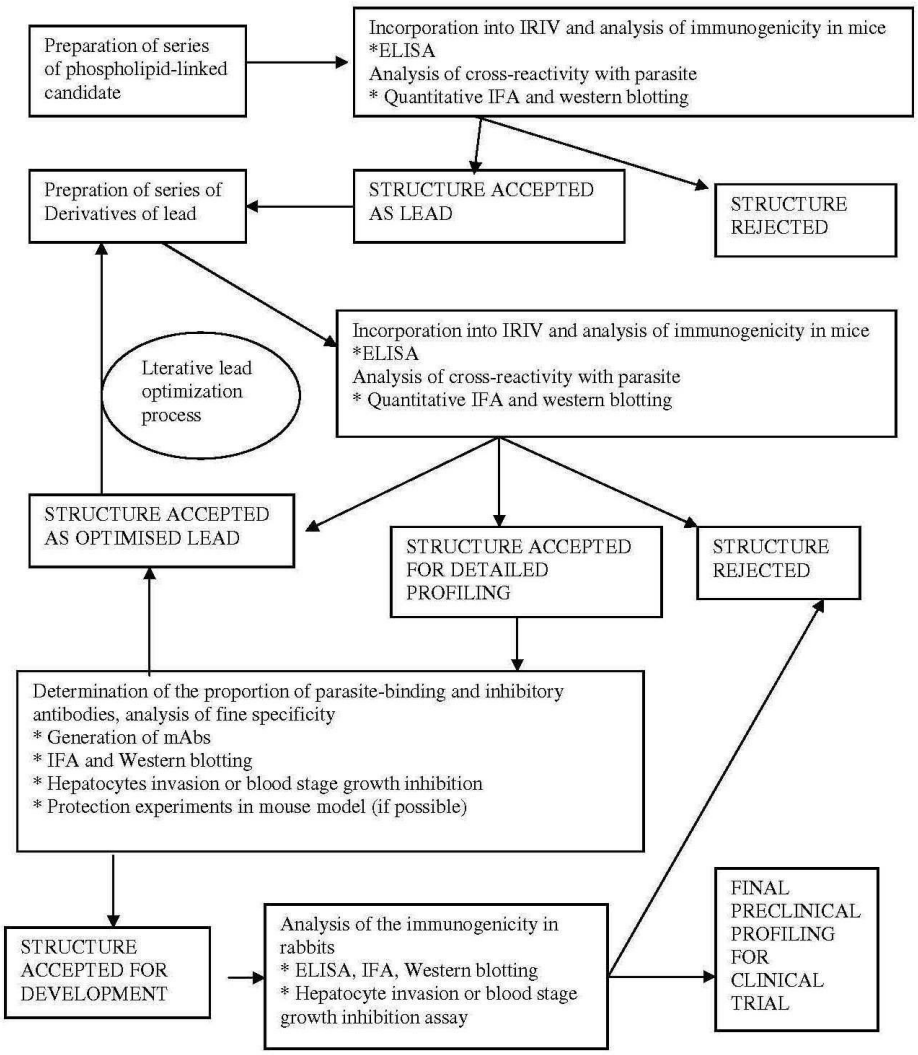

Figure 8. The 'ViroTope' approach for developing optimized mimotopes of parasite antigens (peptidomimetics of malaria surface antigens). Mimotopes are structures that mimic the immunogenic properties of epitopes (from [58]).

ANTIGEN AND ADJUVANT DELIVERY BY VIROSOMES

Adjuvants and antigens from viruses other than influenza can be encapsulated in virosomes or incorporated into their membrane. Virosomes are produced by detergent solubilization of influenza virus, nucleocapsids are sedimented by ultracentrifugation, detergent extracted on bio-beads, and then purified on sucrose gradients. Virosomes contain functionally reconstituted hemagglutinin with receptor-binding and membrane-fusion activity. Thus, virosomes enter cells (also antigen-presenting cells [APCs]) through receptor mediated endocytosis. This way, the contents of the virosomes are released into the cytoplasm where they can enter the major histocompatibility complex (MHC) class I pathway of antigen presentation. Conversely, antigens associated with the virosomal membrane are processed in the endosomes and reach the MHC class II pathway of antigen presentation.

Amphilic adjuvants can be incorporated into the virosomes together with antigens. Lipopolysaccharide incorporated into the membrane of fusion-active virosomes was found to increase antigen-specific immune responses. The first marketed virosome-based vaccine contains HepA inactivated virions associated with the virosomal surface and, thus, targeting the MHC class II pathway [74-80].

TARGETED CYTOSOLIC DELIVERY OF DRUGS THROUGH VIROSOMES

The potential of reconstituted sendal viral envelopes containing only the fusion protein (F-virosomes) was evaluated for the targeted cytosolic delivery of hygromycin B and lysozyme to human hepatoblastoma cells (HepG2) in culture. Interaction of loaded F-virosomes with HepG2 cells resulted in fusion mediated delivery of hygromycin B to the cell

cytoplasm, as was inferred from inhibition of DNA synthesis. The usefulness of F-virosomes with defined specificities as biological carrier for both in vitro and in vivo cytosolic delivery of drugs and proteins has been established [81-83].

CONCLUSION

Virosomes, as vaccine delivery systems, have been shown to be safe and not to engender any antibodies against the phospholipid components. Through the use of virosomes as a delivery vehicle, a number of vaccines have been developed. In humans, virosome-based vaccines containing inactivated hepatitis A and influenza antigens have been found to be efficacious and well-tolerated and have been on the market for several years. Hepatitis B, nucleic acids, cytotoxic drugs, and tetanus and diphtheria toxoids have also been incorporated into virosomes. Further investigations are ongoing in order to define the full potential of virosomes in both prophylactic and immunotherapeutic applications.

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